

A small-angle X-ray scattering investigation of β -lactoglobulins A and B has been carried out. At pH 5.7, where no aggregation occurs, both genetic variants have radii of gyration corresponding to the impinging two-sphere model of Green and Aschaffenburg. The structure of the β -lactoglobulin A tetramer, at pH 4.5, 3°, can be described best by a cubic array of eight spheres.

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Introduction

The molecular state of β -lactoglobulin in the pH region acid to its isoelectric point has been thoroughly examined by one of us²⁻⁷ in studies carried out by a combination of the techniques of electrophoresis, sedimentation, and light scattering. It was shown that at the isoelectric point (pH 5.1-5.2) the kinetic units of both genetic species of this protein (β -lactoglobulins A and B, henceforth referred to as β A and β B) behave hydrodynamically as prolate ellipsoids of revolution with an axial ratio of 2:1 and a molecular weight of close to 36,000.⁵ Between pH 5.1 and 3.7, β A undergoes a reversible association at cold temperatures which is maximal at pH 4.40-4.65.³ The aggregate formed (mol. wt. 144,000)⁴ is a tetramer of the isoelectric species and analyses of its hydrodynamic and thermodynamic behavior have shown it to have a closed compact structure⁶; β B does not undergo this strong tetramerization reaction.⁶ Below pH 3.7 both genetic species dissociate⁷ into two identical half units⁸ of approximately 18,000 molecular weight.⁹ The subunits are single polypeptide chains and behave hydrodynamically like spheres. Green and Aschaffenburg¹⁰ have shown by X-ray crystallography that both β A and β B are composed of nearly spherical units, 18,000 in molecular weight, associated into pairs related by a dyad axis.

While the conclusions drawn from the hydrodynamic and thermodynamic studies on the shape are in good agreement with the crystallographic data, it seemed of great interest to examine the β -lactoglobulins directly in their various states of aggregation in solution.

The method of small-angle X-ray scattering permits the direct examination of the geometry of molecules whose dimensions are in the range of 10-500 Å.¹¹ The recent refinement of the technique permitting the measurement of the scattered intensity on an absolute scale¹²⁻¹⁴ has put within reach a number of molecular parameters not previously accessible to X-ray scattering. The successful application of this technique to

two globular proteins,^{15,16} as well as to polypeptides¹⁷ DNA¹⁸ and RNA,¹⁹⁻²¹ has induced us to employ it in our examination of the β -lactoglobulins and, in particular, our investigation of the geometry of β A tetramerization. This paper will report absolute small-angle X-ray scattering measurements on β -lactoglobulins A and B under conditions at which both are in the isoelectric molecular state and on β A at pH 4.5, 3°, conditions at which the tetramerization reaction has been shown to be maximal. No attempt was made to examine the low pH dissociation, since the equilibrium conditions would require measurements at concentrations too low for the sensitivity of the available equipment.

Experimental and Techniques

Materials and Methods.— β -Lactoglobulins A and B were prepared according to the method of Aschaffenburg²² from the milk of homozygous A/A or B/B cows.

Small-angle X-ray scattering experiments were carried out on β A and β B solutions in a pH 5.7 acetate buffer of 0.1 ionic strength at room temperature, conditions at which no molecular aggregations or dissociations take place. A second series of measurements was made on β A in a pH 4.5 acetate buffer of 0.1 ionic strength at $3 \pm 1^\circ$, which is in the zone of maximal tetramerization.³ All measurements were carried out on rather concentrated isotropic solutions (10-50 g./l.), since at higher dilutions the scattered intensity became too small to be measurable with any significance in the available equipment.

The instrument used was the Guinier type small-angle X-ray scattering apparatus designed and built in the Centre de Recherches sur les Macromolécules in Strasbourg. A detailed description of this device is given elsewhere.²³ A bent quartz crystal monochromator focuses the monochromatic Cu K α_1 radiation on the entrance slit of a Geiger counter. The collimating system consists of a set of horizontal, narrow and long slits. The energy of the direct beam is measured by using a set of calibrated Ni filters. Each experiment consists of measuring the angular distribution, $I(s)$, of the scattered intensity of the solution and the energy, E_0 , of the incident beam, as well as making identical measurements on the same cell filled with solvent. The scattering function, $j_n(s)$ (defined exactly below), is then calculated according to the relation

$$j_n(s) = \left[\frac{I(s)}{\nu \eta E_0} \right]_{\text{solution}} - \left[\frac{I(s)}{\nu \eta E_0} \right]_{\text{solvent}} \quad (1)$$

where ν is a theoretical constant and η is the thickness of the samples, expressed as the number of electrons per cm.². Subtraction of the "blank" eliminates the scattering due to solvent, air, and cell windows.

The cell thickness was measured by a special device consisting of two traveling microscopes. The cell itself consisted of a flat glass (or plastic) spacer, ca. 1 mm. in thickness, sandwiched between two thin mica windows. The total capacity of the cell was 0.35 ml. For measurements carried out at 3°, the temperature was controlled by circulating a thermostated water-methanol mixture through a specially designed cell holder.

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TABLE I
EXPERIMENTAL RESULTS

	βA , pH 5.7						βB , pH 5.7					
1 c , g./l.	12.4	22.8	34.5	45.8	56.9		9.2	18.3	27.5	36.6	45.8	
2 $j_n(0)$	0.246	0.465	0.640	0.865	1.100		0.193	0.367	0.592	0.748	0.989	
3 R_a , Å.	22.2	20.9	21.2	21.8	21.8		21.7	21.8	22.4	21.9	22.2	
4 $i_n(0)$	11.18	19.88	27.81	38.63	49.17		8.59	16.17	27.16	33.53	44.97	
5 M_w , g.	36,770	35,640	33,070	34,680	35,570		38,020	36,190	40,370	37,530	40,340	

Protein concentrations were measured with a Zeiss ultraviolet spectrophotometer at 278 m μ , using an absorptivity value of 0.96 l. cm.⁻¹ g.⁻¹ for both βA and βB .³ pH measurements were carried out at room temperature using a glass electrode.

The electron density of the solvent was calculated to be 0.335 electron-Å.⁻³ at 25° and 0.336 electron-Å.⁻³ at 3°. The value of the protein partial specific volume, \bar{V} , used was that reported by Pedersen²⁴ (0.751 ml.-g.⁻¹ at 25°). The value of \bar{V} at 3°, calculated from this with the mean temperature coefficient for proteins,²⁶ was found to be 0.743 ml.-g.⁻¹. From the amino acid composition,^{26,27} the ratio between the mass of a β -lactoglobulin molecule and its number of electrons was calculated to be 1.87, i.e., 0.322×10^{24} electrons per gram. This results in values of ψ (the electron partial specific volume of the solute) of 2.33 Å^3 -electron⁻¹ at 25° and 2.31 Å^3 -electron⁻¹ at 3°. The resulting relation between the electron concentration, c_e , expressed as the ratio of the number of electrons of the solute to that of the solution, and the concentration, c , expressed in grams of solute per gram of solution, is

$$c_e = 0.962c/(1 - 0.038c) \quad (2)$$

Interpretation.—The working equations and the notation used were those derived by one of us^{12,15} for globular particles in the case of an "infinite slit" collimation. The principal relations used in calculating the molecular parameters will be briefly summarized.

In the case of infinite slit collimation the results of any experiment are expressed in terms of the normalized scattering function $j_n(s)$ (see ref. 15) that depends only on the structure of the sample. If all the molecules of the solute are identical, their mass and radius of gyration can be determined. In order to perform these calculations $j_n(s)$ can be conveniently decomposed^{11,12} into

$$j_n(s) = j_n(0) \exp\left(-\frac{4\pi^2}{3} R_a^2 s^2\right) + \varphi(s) \quad (3)$$

$$s = \frac{2 \sin \theta}{\lambda}$$

where $j_n(s)$ (see eq. 1) is the normalized scattered intensity at the angle corresponding to the given value of s , $j_n(0)$ is that function extrapolated to zero angle, R_a is the apparent radius of gyration,²⁸ λ is the wave length of the incident radiation, 2θ is the angle formed between the incident and scattered beams, and $\varphi(s)$ is a residual function expressing the difference between the gaussian part of eq. 3 (known as the Guinier law) and the scattering actually observed. The mass of the particle, m , expressed in number of electrons in the particle, is given by

$$m = i_n(0)(1 - \rho_0\psi)^{-2}c_e^{-1} \quad (4)$$

where ρ_0 is the electron density of the solvent and

$$i_n(0) = 2 \sqrt{\frac{\pi}{3}} j_n(0) R_a - \frac{1}{\pi} \int_0^\infty s^{-2} \varphi(s) ds \quad (5)$$

The true radius of gyration of one molecule of solute can be obtained from R_a by

$$R_0^2 = \frac{R_a^2 + \left[\frac{9\sqrt{3}\pi}{16\pi^4} \int_0^\infty s^{-4} \varphi(s) ds \right] [j_n(0)R_a]^{-1}}{1 - \left[\frac{\sqrt{3}\pi}{2\pi^2} \int_0^\infty s^{-2} \varphi(s) ds \right] [j_n(0)R_a]^{-1}} \quad (6)$$

Equations 4 and 6 are valid only if correlations between the solute molecules are negligible. The effects of the correlations can be practically eliminated by extrapolating to zero concentration the apparent values of m and R_0 calculated at various concentrations. Furthermore, in a three-component system (such as water, protein, and buffer salts), the extrapolated value of m is the sum of the true molecular weight and a nonextrapolating contribution from the thermodynamic interaction between the

macromolecular and small solutes.^{29,30} Although the effect of such interactions can be large,³¹ it has been shown^{29,30} that for proteins dissolved in a buffer solution (such as the present case) it is smaller than the normal experimental error of measurement and can be neglected.

$j_n(0)$ and R_a can be determined by plotting $\log j_n(s)$ as a function of s^2 (the Guinier plot).¹¹ For small s , a straight line is obtained: its intercept is $j_n(0)$, while its slope is proportional to R_a^2 ; $\varphi(s)$ can then be calculated by subtracting the gaussian function from $j_n(s)$ (eq. 3).

If the electron density of the solute can be assumed to be uniform, several additional parameters can be determined.^{12,15} This particular distribution of the electron density results in a simple mathematical form of $j_n(s)$ at large s .¹⁵

$$\lim_{s \rightarrow \infty} s^2 j_n(s) = A + \delta^* s^3 \quad (7)$$

where A and δ^* are constants.

In this case it is possible to determine the difference ($\Delta\rho = \rho_1 - \rho_0$) between the electronic density, ρ_1 , of the solute and that, ρ_0 , of the solution, the volume, v_1 , of a single solvated molecule of the solute, the ratio S_1/v_1 , where S_1 is the external surface of the same particle, and its degree of solvation, H .

Results and Discussion

pH 5.7.—The data obtained with βA and βB at pH 5.7 were treated in accordance with the Guinier law (eq. 3), i.e., the intensity $j_n(s)$ was plotted logarithmically as a function of s^2 . Very similar results were obtained for both genetic variants. The βB plots are shown in Fig. 1. As can be seen, the points fall on straight

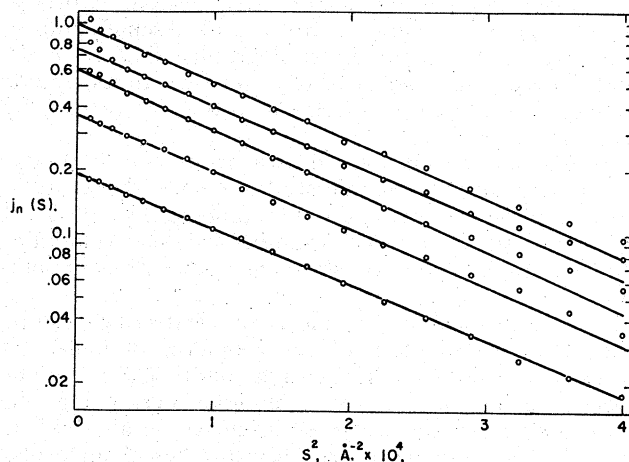


Fig. 1.—Small-angle X-ray scattering data for βB in pH 5.7, acetate buffer ($\Gamma/2 = 0.1$). The experimental points are for protein concentrations of (from top to bottom): 45.8, 36.6, 27.5, 18.3, and 9.2 g./l.

lines at low values of s at all concentrations studied. The slight upward deviation of the points at the lowest angles for the highest protein concentrations may indicate the presence of minute amounts of strongly aggregated denatured material. At higher angles, there is a general upward deviation of the experimental points, reflecting the term $\varphi(s)$ (see eq. 3). From the slopes of the straight line portions of these curves, R_a was calculated, and, from the intercepts, $j_n(0)$. These values are listed in lines 2 and 3 of Table I.

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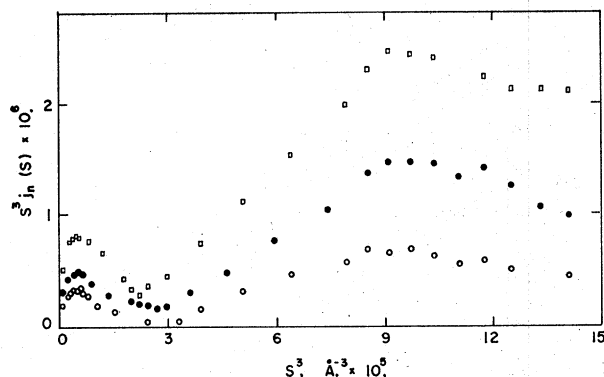


Fig. 2.— $s^3 j_n(s)$ vs. s^3 plot for β B at pH 5.7: \square , 45.8 g./l.; \bullet , 27.5 g./l.; \circ , 18.3 g./l.

The values of $i_n(0)$ and M_c^{32} (eq. 5 and 4) are listed in lines 4 and 5. The contribution of $\varphi(s)$ to R_0 and M was found to be smaller than 1% and can be neglected. Thus, within experimental error, the values of R_a can be equated to the apparent true radii of gyration at each protein concentration ($R_{0,c}$).

The values of R_a and M listed in Table I were plotted as a function of concentration. Their extrapolation to zero concentration yields the actual molecular parameters within the limits of the assumption discussed above. These extrapolated values are listed in Table II and are found to be essentially identical for the two proteins.

TABLE II

MOLECULAR PARAMETERS OF THE β -LACTOGLOBULINS AT pH 5.7

	β A	β B
$R_0 = R_a, \text{\AA}$	21.6 ± 0.4	21.7 ± 0.2
\overline{M}_w	$36,600 \pm 1000$	$36,900 \pm 1100$

The molecular weights of 36,600 and 36,900 agree well within experimental error and are in excellent agreement with values obtained by various other physical and chemical techniques.^{26,27,33-36}

The radius of gyration is found to be consistent with the two-sphere model of β -lactoglobulin proposed by Green and Aschaffenburg.¹⁰ For a structure composed of two identical unimpinging spheres, the radius of gyration, R_2 , is related to the radius of a single spherical subunit, r_1 , by

$$R_2^2 = \frac{3}{5}r_1^2 + a^2 \quad (8)$$

where $2a$ is the distance between the centers of the two spheres. In the case of impinging spheres ($2a < 2r_1$), this equation becomes

$$R_2^2 = \frac{3}{10} \frac{4r_1^5 + 5r_1^4 a - a^5}{(r_1 + a)^2(2r_1 - a)} + a^2 \quad (9)$$

Using the experimental radius of gyration, $R_2 = 21.7 \text{ \AA}$, and assuming that the two spheres touch only at one single point ($r_1 = a$), we obtain $r_1 = 17.2 \text{ \AA}$ and $2a = 34.4 \text{ \AA}$. Green and Aschaffenburg¹⁰ have found r_1 to be 17.9 \AA . By impingement of two spheres of this radius by 2.3 \AA , as suggested by Green and Aschaffenburg, i.e., setting $2a = 33.5 \text{ \AA}$, we calculate $R_2 = 21.8 \text{ \AA}$. Thus, the experimental radius of gyration ($R_2 = 21.7 \pm 0.3 \text{ \AA}$) of the 36,000 molecular weight species of β -lactoglobulin in solution is found to be in good agreement with the Green and Aschaffenburg crystallographic model.

(32) The subscript c refers to the apparent molecular parameter, measured at concentration c .

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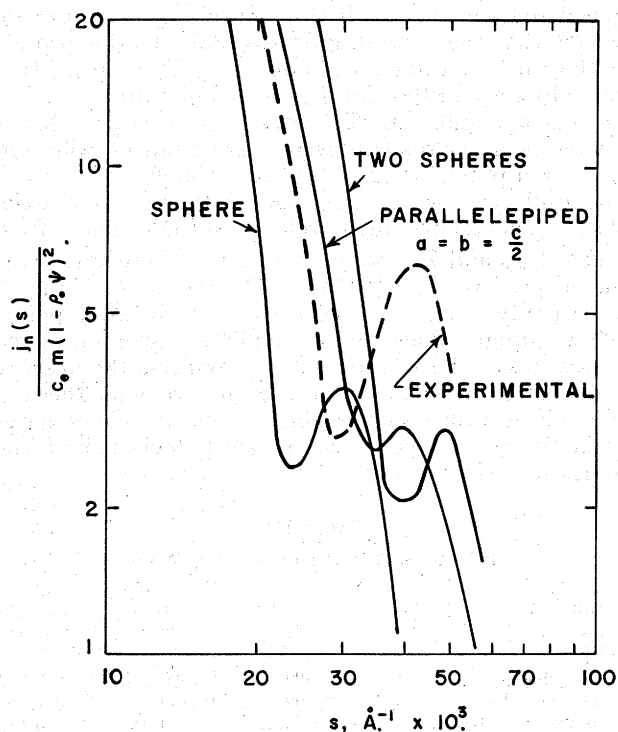


Fig. 3.—Normalized scattering of β -lactoglobulins in the higher angular range: -----, experimental curve; ———, theoretical curves calculated for various models.

In order to calculate $\Delta\rho$, S_1/v_1 , and v_1 , it is necessary to evaluate the constants of eq. 7. In the case of the β -lactoglobulins at pH 5.7, the function $s^3 j_n(s)$ was found to fluctuate over the entire measurable range of angles (up to $s = 50 \times 10^{-3} \text{ \AA}^{-1}$). As a result, the straight line described by eq. 7 cannot be established. The variation of this function with s^3 is shown in Fig. 2 for three concentrations of β B. This complication has precluded the evaluation of the above-listed parameters at pH 5.7.

The presence of a pronounced secondary maximum (at $s = 43 \times 10^{-3} \text{ \AA}^{-1}$) and a minimum (at $s = 30 \times 10^{-3} \text{ \AA}^{-1}$) in the scattering curve indicates that the molecules must be either almost spherical or that their shape is complex. In such case it is sometimes possible to determine the shape of the molecule by direct comparison of the position of maxima and minima in the scattering curve with those found in theoretical curves calculated for various models. Results of such calculations (see Appendix) for a sphere, a parallelepiped and two touching spheres, having radii of gyration of 21.7 \AA , are compared with properly normalized experimental data in Fig. 3. It is found that none of the models gives a maximum and a minimum at the same angles as found experimentally. Thus, it appears that under the experimental conditions β -lactoglobulin cannot be described in terms of these simple models. In all probability the molecule has a complex shape and possibly a nonuniform internal structure. This observation can perhaps be related to that of Tanford, *et al.*,³⁷ who have reported that, under certain conditions, both β -lactoglobulins can undergo a reversible conformational change liberating to titration a buried carboxyl group. This suggests the presence of a certain degree of flexibility and looseness in the β -lactoglobulin structure. However, the agreement of the experimental radius of gyration with the Green and Aschaffenburg model, when

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considered together with the hydrodynamic data,⁵ indicates that the over-all gross structure of this protein in the isoelectric region is not far different from that found by these authors in the crystalline state.

β -Lactoglobulin A Tetramer.—Small-angle X-ray scattering measurements were carried out on solutions of β A in a pH 4.5, $\Gamma/2 = 0.1$ acetate buffer at $3 \pm 1^\circ$. The results are presented in Fig. 4 in the form of Guinier plots. The points align well on straight lines, from which $\overline{M}_{w,c}$ and $\overline{R}_{a,c}$ were obtained. These intercepts and slopes are reported in Table III, lines 5 and 3, respectively. The fact that the experimental points follow straight lines to the smallest angles measured (down to $s = 1.75 \times 10^{-3} \text{ \AA}^{-1}$) confirms the absence of higher aggregates deduced from previous thermodynamic experiments⁶; the presence of such aggregates would have resulted in an upward deviation of the points at the lowest angles.¹¹

TABLE III
EXPERIMENTAL RESULTS, β A, pH 4.5

1 c , g./l.	19.8	37.0	47.2
2 $i_n(0)$	0.728	1.435	1.770
3 \overline{R}_a , \AA .	33.7	32.4	33.1
4 $i_n(0)$	50.22	94.9	120.3
5 \overline{M}_w , approx.	97,300	99,000	98,300
6 \overline{M}_w , calcd.	106,800	116,700	119,200
7 $10^7(2B_0/M_m)$, l.-mole-g. ⁻²	0.47	0.41	0.38
8 R_T (\overline{M}_w , approx.), \AA .	35.6	34.0	34.9
9 R_T (from \overline{M}_w , calcd.), \AA .	35.0	33.3	33.8

Since, under the conditions of the experiment, the protein exists in a state of equilibrium between monomer and tetramer, the values of the molecular parameters of Table III are average ones. They can be evaluated in terms of the known thermodynamic parameters of the $4\beta A \rightleftharpoons \beta A_4$ reaction. The equilibrium constant (K_a) at pH 4.5 and 3° , determined from light scattering data, is $2.8 \times 10^{11} \text{ l.}^3\text{-moles}^{-3}$.⁴ Using this value of K_a , \overline{M}_w was calculated, and its values are listed in line 6 of Table III. Comparison with the experimental values of \overline{M}_w , approx. (line 5) reveals a lack of agreement between the two sets of values. It should be remembered, however, that the calculated value, \overline{M}_w , calcd., corresponds to the ideal situation of eq. 4, which is strictly valid only in the absence of all interactions, i.e., at infinite dilution in a two-component system. At finite protein concentration, in the absence of interactions with the buffer salt components, the relation between $i_n(0)$ and molecular weight has the form

$$\overline{m}_w = \frac{i_n(0)}{c_e(1 - \rho_0\psi)^2} (1 + A\overline{m}_w c_e) \quad (4a)$$

where A is the second virial coefficient expressed in electron units. From the difference between \overline{M}_w , calcd. and \overline{M}_w , approx., values of the second virial coefficient were calculated. For ease of comparison with other systems, these are listed in the usual form for light scattering (see, for example, ref. 4) in line 7 of Table III. Under similar conditions, light scattering measurements on β -lactoglobulin have resulted in a value of $2B_0/M_m$ of $0.50 \times 10^{-7} \text{ l.-mole-g.}^{-2}$ at concentrations near 50 g./l. The agreement between these values, obtained in each case from the difference between two large numbers, is sufficiently good to ascribe the difference between \overline{M}_w , calcd. and \overline{M}_w , approx. to the contribution of thermodynamic interaction between the protein molecules. It should be remarked further that a second virial coefficient of $(0.4\text{--}0.5) \times 10^{-7} \text{ l.-mole-g.}^{-2}$ is very low and would make a contribution well within experimental error in the usual

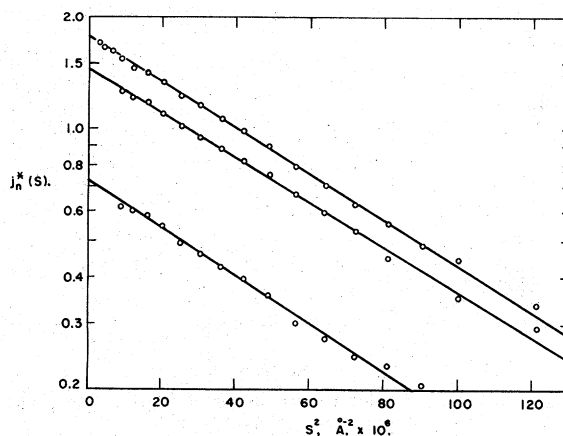


Fig. 4.—Small-angle X-ray scattering data for β A in pH 4.5, acetate buffer ($\Gamma/2 = 0.1$) at 3° . The experimental points are for protein concentrations of (from top to bottom): 47.2, 37.0, and 19.8 g./l.

range of concentrations in which protein solutions are studied ($<10 \text{ g./l.}$).

In this work the parameter of particular interest is the radius of gyration, since it yields direct information on the geometry of the aggregate and permits comparison with tetramer models. The average radius of gyration is given by¹¹

$$\overline{R}^2 = \frac{\sum_i n_i m_i^2 R_i^2}{\sum_i n_i m_i^2} \quad (10)$$

where n_i is the number of particles of type i , m_i is the number of electrons contained in particle i , and R_i is its radius of gyration. The experimental values of the apparent average radii of gyration, \overline{R}_a , are listed in line 3 of Table III. The true radius of gyration, R_0 , was calculated for the most concentrated solution, using eq. 3 after evaluation of the factor $\varphi(s)$ as described above. The correction was found to be of the order of 1%, both for R_0 and $i(0)$, or well within experimental error.

In the case of β -lactoglobulin A only two molecular species are present,⁶ namely the monomer of molecular weight 36,600 (see Table II) and the tetramer of molecular weight 146,400. The radius of gyration of the monomer is 21.7 \AA . Since the weight average molecular weight is known, the number of monomer and tetramer particles can be calculated at each concentration. From this, the experimental value of \overline{R} and the radius of gyration of the monomer, the radius of gyration of the tetramer, R_T , can be calculated. This was done, using as \overline{M}_w both the experimental value, \overline{M}_w , approx., and the value calculated from the equilibrium constant, \overline{M}_w , calcd. The values of R_T obtained at each concentration are listed in the last two lines of Table III. The two sets of values are very close to each other and remove the uncertainty resulting from the contribution of virial effects. Furthermore, due to the high average of \overline{R} , R_T in each case turns out to have a value close to \overline{R}_a .³⁸

Using the radius of gyration of the tetramer and the known dimension of the monomer, a comparison was made with various geometric configurations of the tetramer. Radii of gyration (averaged over the three axes of rotation of the particle) were calculated for

(38) It should be pointed out that the measured radius of gyration is also a concentration-dependent quantity, the true value being obtained by extrapolation to zero concentration. In a study on a system in rapid dynamic equilibrium, such as the present one, extrapolation is impossible. Examination of the values of R_T , given in Table III, shows no trend with concentration, indicating that the virial effect is not large. The maximum error in R_T is estimated to be not greater than 10%.

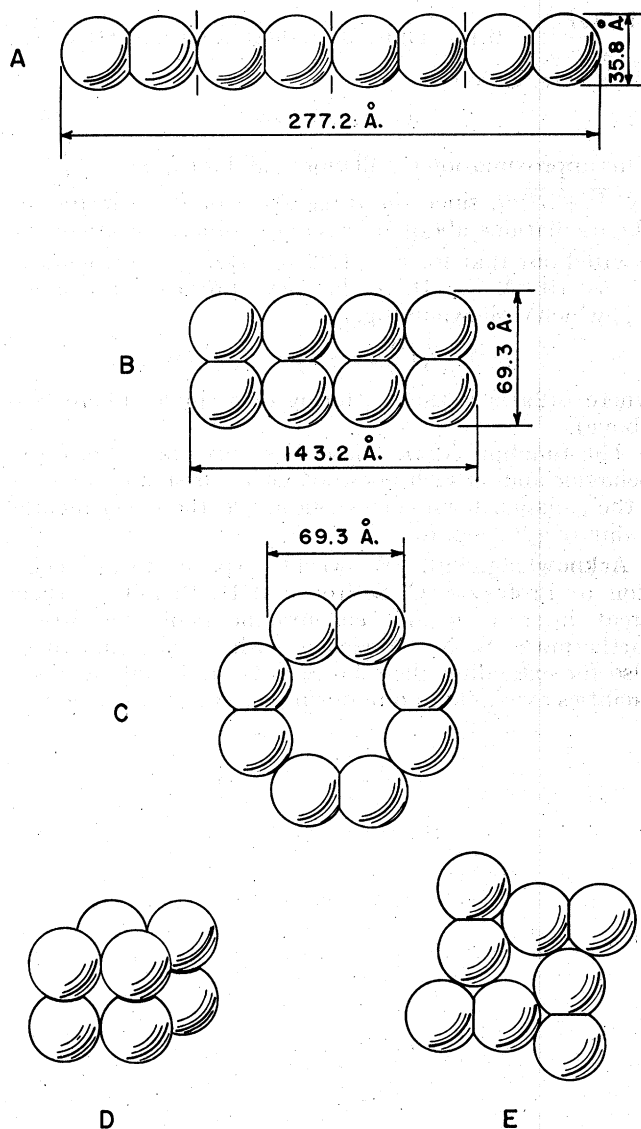


Fig. 5.—Various possible models of βA tetramer: (A) end-to-end aggregate; (B) side-by-side aggregate; (C) closed ring; (D) compact cubic array; (E) crystallographic $P22_12_1$ structure. The monomeric units are represented in terms of the Green and Aschaffenburg model.

different possible models of the tetramer. These included: a sphere with four times the volume of the monomer, an end-to-end aggregate (Fig. 5A), a side-by-side aggregate (Fig. 5B), and three types of closed structure aggregates, a ring with the intermolecular bonds located close to the long axis of the monomer (Fig. 5C), a tightly closed cubic array with the bonds located along the short axis of the molecule (Fig. 5D), and the $P22_12_1$ crystallographic model, composed here of four monomers (Fig. 5E). Knowing the radius of gyration of the Green and Aschaffenburg monomer and the positions of the centers of the four monomeric units of the tetramer, it is possible to calculate the radii of gyration of these models. These models can serve as reasonable approximations to the various types of aggregates, since the purpose of the calculation is to eliminate those which differ greatly in radius of gyration from the experimental entity, even though the protein molecule in reality must be irregular in shape and no simple geometric model is a correct representation of the actual configuration.

The calculated values of R_T for the various models are compared with the average experimental radius of gyration in Table IV. It is possible to eliminate im-

TABLE IV
CALCULATED RADII OF GYRATION OF VARIOUS TETRAMER MODELS

Model	R_T , Å
Sphere	27.7
End-to-end aggregate (A)	81.0
Side-by-side aggregate (B)	47.1
Flat ring (C)	48.7
Cube (D)	35.4
$P22_12_1$ model (E)	40.5
Experimental value	34.4 ± 0.4

mediately the end-to-end, side-by-side, and closed flat ring aggregate models as being much too high. The spherical model can also be eliminated because of a too small value of R_T . The two compact structures (D and E) observed in the crystal state are both sufficiently close to the experimental value to be consistent with it, and a definite choice between the two becomes impossible on the basis of X-ray scattering alone, although the cubic array model results in the best agreement. Analysis of the structural differences between βA and βB ,^{39,40} together with the differences in their physical-chemical behavior,²⁻⁸ have shown⁴¹ that the pair of tetramerization sites on each 36,000 molecular weight monomer must be symmetrically arranged about the dyad axis of symmetry. Structure $P22_12_1$ involves a bond between a site located on the polar axis of one monomer with a site located close to the area of contact between the 18,000 molecular weight subunits. This would violate the requirements of a dyad axis of symmetry and the earlier conclusion that the two subunits of the monomer must be identical.⁸ On the basis of these considerations the $P22_12_1$ structure could be eliminated as that of the tetramer. Thus, the present study, when combined with earlier considerations, suggests that the β -lactoglobulin tetramer has the geometric structure of a cubic array of eight spheres, these being joined by two types of bonds—four joining the 18,000 molecular weight subunits to form the iso-electric species, and four joining the last to give the low temperature aggregate.

Appendix

Calculation of Scattering Curves for Various Models.

—The scattering curves for three models have been calculated, namely for a sphere, a parallelepiped, $a \times a \times 2a$, and a structure consisting of two spheres touching at one point. These three bodies were assumed to have a constant internal electron density; their radius of gyration has been set equal to the experimental value of $R_0 = 21.7$ Å. The scattering curve, normalized to $i(0) = 1$ (see ref. 12), was calculated for each case

$$j(s) = \frac{j_n(s)}{c_0 m (1 - \rho_0 \psi)^2} \quad (11)$$

Case 1. Single Sphere of Radius r .—The function $j_0(s)$, normalized to $j_0(0) = 1$, has been calculated by Schmidt.⁴²

For a sphere of radius r , we find easily that

$$j_n(0) = (16\pi^2/15)r^5 \quad (12)$$

and

$$i_n(0) = (16\pi^2/9)r^6 \quad (13)$$

Combining these two relations gives

$$j(s) = (3/5r)j_0(s) \quad (14)$$

Case 2. Rectangular Parallelepiped, $a \times a \times 2a$.—The functions $i(x = 2\pi as)$, normalized to $i_n(0) = 1$,

(39) E. B. Kalan, W. G. Gordon, J. J. Basch, and R. Townend, *Arch. Biochem. Biophys.*, **96**, 376 (1962).

(40) R. Townend, Abstracts, 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept., 1961, p. 25C.

(41) S. N. Timasheff and R. Townend, *J. Dairy Sci.*, **45**, 259 (1962).

(42) P. W. Schmidt, *Acta Cryst.*, **8**, 772 (1955).

have been calculated for various models of rectangular parallelepipeds by Mittelbach and Porod.⁴³ In the present study the scattering curve for infinite slit collimation, $j(s)$, was calculated for the $a \times a \times 2a$ case, by decomposing $i(s)$ into its asymptotic form and the oscillations about this average function.

$$i(x = 2\pi as) = (5\pi/x^4) + u(x) \quad (15)$$

Then

$$\begin{aligned} j(2\pi as) &= 2 \int_0^\infty i(\sqrt{s^2 + t^2}) dt \\ &= \frac{5\pi}{4x^3} + \frac{1}{\pi a} \int_0^\infty u \sqrt{x^2 + y^2} dy \end{aligned} \quad (16)$$

The second term was evaluated by graphical integration.

Case 3. Two Sphere Model.—The two spheres touch at a single point. The scattering of such a model, normalized to $i(0) = 1$, can be readily calculated for point source collimation

$$i(s) = \frac{1}{2} \left(1 + \frac{\sin 4\pi rs}{4\pi rs} \right) \phi^2(2\pi rs) \quad (17)$$

where $\phi^2(2\pi rs)$ is the scattering of a single sphere of radius r , normalized to $\phi^2(0) = 1$. For $4\pi rs \geq 10$,

(43) P. Mittelbach and G. Porod, *Acta Phys. Austriaca*, **14**, 186 (1962).

$\left| \frac{\sin 4\pi rs}{4\pi rs} \right| \leq 0.1$. Thus, as a first approximation, for large $4\pi rs$, we have

$$i(s) \simeq \frac{1}{2} \phi^2(2\pi rs) \quad (18)$$

This approximation is still more valid for $j_n(s) = 2 \int_0^\infty i_n(\sqrt{s^2 + t^2}) dt$, since the integration of $i(s)$ smears out the oscillations about its average value. It should be pointed out that for $r = 17.2 \text{ \AA.}$, $4\pi rs \geq 10$ when $s > 45 \times 10^{-3} \text{ \AA.}^{-1}$. It is this asymptotic behavior of $j(s)$ which is shown in Fig. 3.

$$j(s) = \frac{1}{2} \left(\frac{3}{5} j_0(2\pi rs) \right) = \frac{3}{10} j_0(2\pi rs) \quad (19)$$

where $j_0(2\pi rs)$ is the scattering of a single sphere (see above).

The function $j(2\pi rs)$ describes only the asymptotic behavior and as such does not yield for small values of s the gaussian form corresponding to the experimental radius of gyration, R_0 .

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